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Plasmid-Mediated Quinolone Resistance in *Pseudomonas putida* Isolates from Imported Shrimp▽

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Fourteen quinolone-resistant *Pseudomonas putida* isolates were recovered from imported frozen shrimp sold in the United States. Two isolates harbored plasmids with *qnrA* and *qnrB* genes. PCR and DNA sequencing of quinolone resistance-determining regions identified novel substitutions in GyrA (His139→Glu and Thr128→Ala) and GyrB (Thr442→Asn, Gly470→Ala, and Ile487→Pro) and previously reported substitutions in GyrB (Asp489→Glu) and ParC (Thr105→Pro).

The administration of quinolones, including nalidixic acid, oxolinic acid, enrofloxacin, norfloxacin, and ciprofloxacin, is a common practice in the shrimp-farming industry of developing countries, raising concern over the generation of multiresistant pathogenic bacteria (7, 11, 17). *Pseudomonas putida* is a fast-growing organism that is found in most temperate soil and water habitats and causes opportunistic infections in humans (2, 16, 19, 21). This study describes the isolation, characterization, and detection of quinolone resistance mechanisms among *P. putida* isolates from imported shrimp.

Fourteen isolates of *P. putida* were isolated from a total of 10 samples of farm-raised, frozen, whole raw shrimp (*Penaeus* spp.) imported from India and purchased in retail stores (Little Rock, AR). One gram of shrimp was taken from three individual bags and homogenized in a stomacher with 10 ml of LB broth supplemented with 10 μg/ml of nalidixic acid. The homogenate was enriched overnight at 37°C, and 100 to 200 μl was subcultured onto TSAII plates (Becton Dickinson, Franklin Lakes, NJ). Isolates were confirmed as *P. putida* using VITEK 2 Gram-negative identification cards (bioMérieux, Durham, NC).

Pulsed-field gel electrophoresis (PFGE) analysis was performed using the Centers for Disease Control and Prevention procedure for *Salmonella* (http://www.cdc.gov/pulsenet/protocols.htm) to assess the genetic relatedness among the *P. putida* isolates recovered from the shrimp. Upon examination of PFGE patterns from SpeI restriction digests of DNA from the 14 *P. putida* strains, 10 clusters, each containing 14 to 20 restriction fragments, were apparent (Fig. 1).

The antimicrobial MICs for *P. putida* isolates were determined using the Sensititre automated antimicrobial susceptibility system according to the manufacturer's instructions (25) and interpreted based on the CLSI criteria (5). When the β-lactam antimicrobial MICs were determined, all isolates were found resistant to cefoxitin, amoxicillin-clavulanic acid, ceftiofur, and ampicillin, and 8 of the 14 were resistant to ceftriaxone. When the aminoglycoside MICs were determined, the most isolates were found to be resistant to kanamycin (10/14 isolates), followed by streptomycin (9/14), amikacin (9/14), and gentamicin (1/14). When the remaining antimicrobial MICs were determined, all isolates were found resistant to sulfisoxazole and 12 of the 14 were resistant to chloramphenicol, tetracycline, and trimethroprim-sulfamethoxazole. All isolates were resistant to nalidixic acid (≥32 μg/ml). Isolate PP3 exhibited intermediate resistance to ciprofloxacin (2 μg/ml). All other isolates possessed various susceptibilities to ciprofloxacin, ranging from 0.25 to 1 μg/ml. MICs determined for nalidixic acid and ciprofloxacin corresponding to the *P. putida* isolates grouped by PFGE patterns are displayed in Table 1.

Limited studies with clinical *P. putida* isolates report that fluoroquinolone resistance involves point substitutions in DNA gyrase (*gyrA* and *gyrB*) and topoisomerase IV (*parC*) (12). The amplification of quinolone resistance-determining regions (QRDRs) of *gyrA* (~400 bp), *gyrB* (~370 bp), and *parC* (~180 bp) was carried out with previously published primers and amplification conditions (1). DNA sequences were analyzed using the Pseudomonas Genome Database BLAST (28).

Among the QRDRs, a majority (11/14) of the isolates contained a single replacement in GyrA (His-139→Glu). Isolate PP10 possessed an additional substitution in GyrA (Thr-128→Ala). For GyrB, a single substitution in PP3 (Asp-489→Glu) and three substitutions in PP16 (Thr-442→Asn, Gly-470→Ala, and Ile-487→Pro) were identified. Four of the isolates (PP21, PP24, PP25, and PP3) showed the replacement of Thr-105 by Pro in ParC. The substitutions detected in *parC* (Thr-105→Pro) and *gyrB* (Asp-489→Glu) agreed with findings from a previous study (12). Our findings of amino acid changes in GyrA and GyrB differed somewhat from those of the previous study, which reported substitutions such as Thr-83→Ile and Ser-136→Ala in GyrA and Glu-469→Asp in GyrB as possible substitutions contributing to high levels of fluoroquinolone resistance in human clinical isolates (12). Moreover, our findings differed from GyrA substitutions in clinical isolates of *Klebsiella pneumoniae*, *Escherichia coli*, and *Pseudomas aeruginosa*, which have been shown to comprise Ser-83→Ile, Ser-83→Leu, and Thr-83→Ile, respectively (4, 6, 27). A possible

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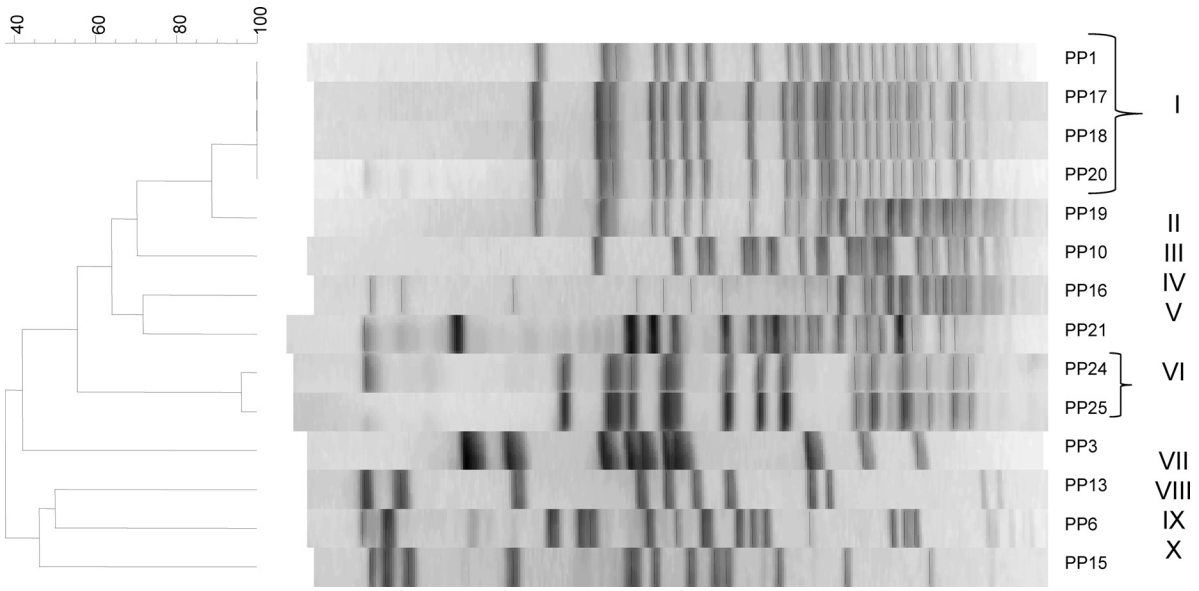

FIG. 1. Dendrogram generated by unweighted-pair group method using average linkage (UPGMA) analysis, using Bionumerics software, showing the results of cluster analysis of PFGE profiles of *Pseudomonas putida* isolates from imported shrimp digested with SpeI. The clusters are marked I through X.

explanation for these discrepancies may be related to the sources of the isolates examined.

Interestingly, among the isolates in this study, PP3 exhibited a higher level of resistance to ciprofloxacin and possessed substitutions in *gyrA*, *gyrB*, and *parC* QRDRs, suggesting that, as found in other *Enterobacteriaceae*, high-level fluoroquinolone resistance may be associated with an increased number of substitutions present in the QRDRs (4, 9).

In 1998, the first plasmid-mediated quinolone resistance gene (*qnr*) was discovered in a *Klebsiella pneumoniae* clinical isolate (18). The *qnr* plasmid mediates low-level quinolone resistance and facilitates selection of higher-level resistance mutations (18). The original *qnr* gene is now designated *qnrA* because two other plasmid-mediated quinolone resistance genes, *qnrB* (14) and *qnrS* (8), possess mechanisms of action similar to that of *qnrA* in reducing fluoroquinolone activity. Plasmid extraction was performed using a QIAprep spin miniprep kit (Qiagen Inc., Valencia, CA) on the 14 *P. putida* isolates, but plasmids were recovered from 2, PP16 and PP19. PCR assays using previously published primers (29) detected *qnr*A and *qnrB* but not *qnrS* in both plasmids. Subsequently, PCR products for *qnrA* and *qnrB* were confirmed by restriction enzyme digestion and nucleotide sequencing.

Shewanella algae, a Gram-negative bacterium of marine and fresh water, was recently identified as a possible natural source of the plasmid-mediated quinolone resistance determinant QnrA

TABLE 1. Characteristics of QRDR substitutions and quinolone MICS for *Pseudomonas putida* isolates from imported shrimp

PFGE pattern	Isolate	MIC (μg/ml) of:		Amino acid change(s)		
		Nal[a]	Cip[b]	GyrA	GyrB	ParC
I	PP1	≥32	1	His139→Glu		
	PP17	≥32	1	His139→Glu		
	PP18	≥32	1	His139→Glu		
	PP20	≥32	1	His139→Glu		
II	PP19	≥32	1	His139→Glu		
III	PP10	≥32	1	Thr128→Ala, His139→Glu		
IV	PP16	≥32	0.25		Thr442→Asn, Gly470→Ala, Ile487→Pro	
V	PP21	≥32	0.25			Thr105→Pro
VI	PP24	≥32	0.25			Thr105→Pro
	PP25	≥32	0.50	His139→Glu		Thr105→Pro
VII	PP3	≥32	2	His139→Glu	Asp489→Glu	Thr105→Pro
VIII	PP13	≥32	1	His139→Glu		
IX	PP6	≥32	1	His139→Glu		
X	PP15	≥32	1	His139→Glu		

[a] The resistance breakpoint for Nal (nalidixic acid) according to CLSI standards (5) is ≥32 μg/ml.
[b] The resistance breakpoint for Cip (ciprofloxacin) according to CLSI standards (5) is ≥4 μg/ml.

(22). Since quinolones are widely administered in the aquaculture of less developed countries, including the shrimp-farming industry (11), it is possible at any concentration of quinolones, including low concentrations, to select for waterborne *S. algae* strains and promote the horizontal transfer of *qnr* genes to other bacteria, including *P. putida* found in water habitats. The generation of resistant pathogens in aquaculture environments is well documented (10, 15, 26, 31), and evidence of transfer of resistance-encoding plasmids between bacteria found in aquaculture environments and humans has been shown (23). Therefore, it can be speculated that the aquatic environment may play a possible role as a reservoir for antibiotic resistance genes.

The transferability of plasmid-mediated quinolone resistance in *P. putida* strains harboring *qnrA* and *qnrB* genes was studied. Plasmids were transferred from *P. putida* to *Escherichia coli* J53 Azi^r by conjugation using sodium azide (100 μg/ml) for counterselection (13). Ceftazidime (10 μg/ml) was used in mating experiments instead of quinolones to avoid selection of quinolone resistance chromosomal mutations and because of the strong association between *qnr* genes and plasmids carrying cephalosporinase genes (3, 18, 20, 24). To determine if quinolone resistance was transferred, the MICs for the donor, recipient, and transconjugant were measured and evaluated with 30-μg nalidixic acid disks.

The MIC of nalidixic acid for transconjugants demonstrated an 8-fold increase, from 4 μg/ml to 32 μg/ml, over the MIC for the recipient *E. coli* strain J53. The MIC of ciprofloxacin for transconjugants demonstrated approximately a 4-fold increase, from 0.06 μg/ml to 0.25 μg/ml, over that for the recipient *E. coli* strain J53. The plasmid carrying *qnrA* and *qnrB* genes provided resistance to ciprofloxacin and nalidixic acid, a finding which agreed with earlier reports (14, 18). The inhibition zones for recipient and donor strains were 18 mm and 8 mm, interpreted as susceptible and resistant, respectively. No zone of inhibition was present for the transconjugant, indicating that the recipient *E. coli* J53 had acquired quinolone resistance. Transconjugants also displayed decreased susceptibility to cefoxitin, chloramphenicol, amoxicillin-clavulanic acid, and ampicillin, suggesting that the plasmid may carry additional antibiotic resistance elements, as shown in previous studies (18, 29, 30).

In summary, the findings of mutations in the bacterial enzymes DNA gyrase and topoisomerase IV and plasmid-borne *qnr* genes among *P. putida* isolates from shrimp in this study support reports that the use of antimicrobial agents in aquaculture might promote an increase in the frequency of antibiotic resistance genes in the microbiota of finfish, crustaceans, shellfish, and the environment.

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